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Determination of Multiple Mycotoxins in Dietary Supplements Containing Green Coffee Bean Extracts Using Ultrahigh-Performance Liquid Chromatography−Tandem Mass Spectrometry (UHPLC-MS/MS)

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Supporting Information

ABSTRACT: An ultrahigh-performance liquid chromatography−tandem mass spectrometry (UHPLC-MS/MS) method for the determination of 34 mycotoxins in dietary supplements containing green coffee bean (GCB) extracts was developed, evaluated, and used in the analysis of 50 commercial products. A QuEChERS-like procedure was used for isolation of target analytes from the examined matrices. Average recoveries of the analytes were in the range of 75−110%. The precision of the method expressed as relative standard deviation was below 12%. Limits of detection (LODs) and limits of quantitation (LOQs) ranged from 1.0 to 50.0 μg/kg and from 2.5 to 100 μg/kg, respectively. Due to matrix effects, the method of standard additions was used to ensure accurate quantitation. Ochratoxin A, ochratoxin B, fumonisin B_1 and mycophenolic acid were found in 36%, 32%, 10%, and 16% of tested products, respectively. Mycotoxins occurred in the following concentration ranges: ochratoxin A, <1.0−136.9 μg/kg; ochratoxin B, <1.0−20.2 μg/kg; fumonisin B_1, <50.0−415.0 μg/kg; mycophenolic acid, <5.0−395.0 μg/kg. High-resolution mass spectrometry operated in full MS and MS/MS mode was used to confirm the identities of the reported compounds.

KEYWORDS: *mycotoxins, green coffee bean extracts, dietary supplements, liquid chromatography−mass spectrometry*

INTRODUCTION

Overweight and obesity represent serious health problems and are among the leading causes of death. Rates of obesity have more than doubled worldwide since 1980.[1] Because of the relatively high cost and small number of prescription weight loss drugs, limited effectiveness, and their potential adverse health effects, the use of dietary supplements is increasingly perceived by consumers as a safer and more natural way to treat overweight and obesity.

Green coffee beans (GCB) and GCB extracts are advertised as representing inexpensive natural ingredients with high potential to induce weight loss, as they contain high concentrations of caffeine and chlorogenic acids.[2] Caffeine has been shown to promote lipolysis in both animals and humans.[3,4] A small, short-term clinical trial conducted by Thom et al.[5] compared weight loss and body fat reduction in 30 subjects randomly assigned to receive instant coffee or chlorogenic acid enriched instant coffee. Significant weight loss and body fat reduction from baseline was reported in the enriched coffee group, but not the regular instant coffee group. Between group differences were not evaluated. Chlorogenic acid along with caffeic and quinic acids were shown to inhibit α-amylase enzymes in vitro.[6] If such inhibition is found to occur in human or animal subjects, the above chlorogenic acids could decrease the breakdown of starch into glucose and lower the caloric intake. More recently, Vinson et al.[7] evaluated the efficacy of a commercial GCB extract in weight loss. Significant reductions in body weight (−8.04 ± 2.31 kg), body mass index (−2.92 ± 0.85 kg/m^2), and percent body fat (−4.44 ± 2.00%) were observed in 16 overweight adults during 22 weeks of treatment. It should be noted that relatively high amounts of GCB extract (high-dose and low-dose levels were 1050 and 700 mg/day, respectively) containing approximately 57% of chlorogenic acids and 2.6% of caffeine were used. As a result of promotion of the above finding in the media, many dietary supplements containing GCB extracts are now widely available.

Like other food commodities, GCB may contain certain concentrations of exogenous contaminants, such as mycotoxins and pesticide residues that can pose a health hazard to consumers. Mycotoxins are toxic secondary metabolites of filamentous fungi that can grow on crops both in the field and/or during processing and storage. Because of the widespread distribution of the toxigenic fungi in the environment, the occurrence of mycotoxins cannot be entirely avoided.[8] Ochratoxin A (Figure 1, **1**) represents the most common mycotoxin found in both green and roasted coffee beans. It is produced mainly by the fungi *Aspergillus ochraceus* and *Penicillium verrucosum*.[9] The contamination of coffee with ochratoxin A is of considerable importance due to its nephrotoxicity, hepatotoxicity and carcinogenicity in animals.[10] Ochratoxin A is also classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans.[11] Several studies focused on screening ochratoxin A concentrations in the commodity green coffee beans have been reported.[12−15] In one of the most extensive screening studies,[12]

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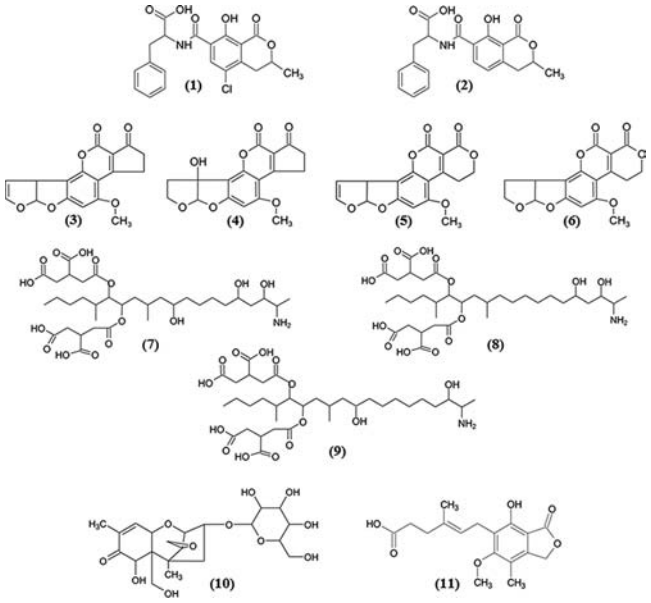

Figure 1. Structures of ochratoxin A (**1**), ochratoxin B (**2**), aflatoxin B_1 (**3**), aflatoxin B_2 (**4**), aflatoxin G_1 (**5**), aflatoxin G_2 (**6**), fumonisin B_1 (**7**), fumonisin B_2 (**8**), fumonisin B_3 (**9**), deoxynivalenol-3-glucoside (**10**), and mycophenolic acid (**11**).

162 GCB samples from Africa, North and South America, and Asia were evaluated. The results showed that ochratoxin A was present in 106 of the 162 samples, with concentrations ranging from 0 to 48 μg/kg. In addition to ochratoxin A, ochratoxin B (Figure 1, **2**) and aflatoxins (Figure 1, **3**–**6**) have also been reported in green and roasted coffee.[16−18] Because commercial GCB-based dietary supplements often contain additional ingredients such as herbal and/or fruit extracts, other mycotoxins may also be present in these products.

Currently, no regulatory limits are set for most mycotoxins in either GCB or dietary supplements containing GCB. The U.S. Food and Drug Administration (FDA) has set an action level for aflatoxins at 20 μg/kg in foods.[19] The European Union (EU) has established maximum limits for ochratoxin A in roasted and instant coffee to 5 and 10 μg/kg, respectively. Regulatory limits for ochratoxin A in GCB will be set by the EU in the future.[20] Ochratoxin A and several other mycotoxins are included in the US FDA compliance program guidance manual, which describes the compliance program that collects monitoring and incidence data to support establishment of regulatory limits.[21]

Mycotoxins are usually determined primarily by methods allowing analysis of a single compound or a small group of similar compounds. For this purpose, highly specific, laborious and time-consuming sample preparation procedures, such as immunoaffinity cleanup, were used.[8,22] In addition, rapid and broad-based methods employing liquid chromatography−mass spectrometry (LC-MS) that enable simultaneous quantitation of multiple mycotoxins at low concentrations in crude extracts, have been developed.[23−25]

In this study, an ultrahigh-performance liquid chromatography−tandem mass spectrometry (UHPLC-MS/MS) method employing procedure based on a QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction protocol was evaluated for determination of 34 mycotoxins in dietary supplements containing GCB. The analyzed mycotoxins included ochratoxin A and B, aflatoxins (aflatoxin B_1, B_2, G_1, G_2), trichothecenes A and B (deoxynivalenol, deoxynivalenol-3-glucoside, nivalenol, zearalenone, HT-2 toxin, T-2 toxin, neosolaniol, 3-acetyldeoxynivalenol, diacetoxyscirpenol), *Alternarium* toxins (alternariol, alternariol-methyl ether, tentoxin), fumonisins (fumonisin B_1, B_2, B_3), enniatins (enniantin A, A_1, B, B_1), beauvericin, and other mycotoxins produced by *Aspergillus* and *Penicillium* species (citrinin, cyclopiazonic acid, mycophenolic acid, penicillic acid, penitrem A, roquefortin C, gliotoxin, sterigmatocystin). The method was applied to a set of 50 dietary supplements available in the US. Positive results were confirmed for identity on the basis of accurate mass measurements and high-resolution (HR) MS/MS spectra with the use of a benchtop quadrupole-orbitrap tandem mass spectrometer. To the best of our knowledge, this is the first report of the analysis and quantitation of mycotoxins in dietary supplements containing GCB.

MATERIALS AND METHODS

Standards and Chemicals. Analytical standards of beauvericin, enniantin A, enniantin A_1, enniantin B, and enniantin B_1 were purchased from Enzo Life Science (Farmingdale, NY, USA). The remaining mycotoxin standards were purchased from RomerLabs (Franklin, MO, USA). The purity of all standards was ≥95.0%. Stock solutions of individual analytes were prepared in acetonitrile at concentrations ranging from 10−1000 μg/mL and combined into mixed standards at 100 and 1000 ng/mL. Diluted standard solutions were stored in a freezer at −25 °C. Deionized water was obtained from an Aqua Solutions purification system (Aqua Solutions Inc., Jasper, GA, USA). LC-MS grade Optima acetonitrile and methanol were supplied by Fisher Scientific (Pittsburgh, PA, USA). Formic acid (≥95%), ammonium formate (≥99.9%), ammonium acetate (≥99.9%), anhydrous magnesium sulfate (≥99.5%), and sodium chloride (≥99.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Test Products and Preparation. Fifty dietary supplements containing GCB extracts were purchased from Internet vendors. Dietary supplements were obtained from different suppliers and at least two bottles from the same lot were purchased for each. An overview of declared compositions and dosage forms of the dietary supplements is provided in Table 1.

The respective dosage forms (i.e., capsules, softgels, and packs of powdered extracts) were opened and the contents removed and thoroughly homogenized prior to preparation using the QuEChERS method.[25,26] The test portion (2.0 ± 0.01 g) was weighed into a 50 mL polypropylene centrifuge tube and 10 mL of water containing 2% (v/v) of formic acid was added. The mixture was briefly hand-shaken and incubated for 20 min under ambient conditions to allow the matrix to absorb the solvent. Ten millliters of acetonitrile was added to the tube and the tube was shaken at 500 rpm for 30 min using a Glas-Col digital pulse mixer (Terre Haute, IN, USA). In the next step, 4 g of anhydrous $MgSO_4$ and 1 g of NaCl were added to the tube. The test portion was then vigorously shaken for 1 min to prevent formation of agglomerates due to hydration of $MgSO_4$ and centrifuged for 7 min at 4,500 rpm. The upper acetonitrile layer was filtered through a 0.22 μm PFTE filter, diluted 1:1 with deionized water, and this test solution was transferred into screw-cap amber autosampler vials with PFTE-lined septa.

UHPLC-MS Conditions. Two LC-MS systems were used in this study. The multimycotoxins analysis of test solutions was performed with a Prominence UFLC XR chromatographic system (Shimadzu, Kyoto, Japan) interfaced with an AB SCIEX 4500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer equipped with a TurboIon electrospray (ESI) ion source (AB SCIEX, Toronto, ON, Canada). A Q Exactive quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an Accela U-HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) via an HESI-II ESI source was used for confirmatory analyses of the target mycotoxins.

Table 1. Dietary Supplements Containing Green Coffee Bean (GCB) Extracts

product	description	serving size (capsules)	GCB extract per serving (mg)	other ingredients	maximum recommended daily intake (capsules)
1	GCB extract	1	800	cellulose	2
2	GCB extract	1	400	cellulose, magnesium stearate, silica	3
3	GCB extract	1	400	cellulose, magnesium stearate, silica	1
4	pure GCB extract	2	800	cellulose	2
5	pure GCB extract	1	800	cellulose	2
6	GCB extract	1	200	cellulose, magnesium stearate, silica	2
7	pure GCB extract	1	800	gelatin	2
8	GCB extract	1	400	cellulose, magnesium stearate, silica	2
9	GCB extract	1	400	cellulose, rice bran, silica	2
10	GCB extract	1	800	cellulose	2
11	GCB extract	1	350	gelatin, magnesium stearate	3
12	pure GCB extract	2	800	cellulose, gellan gum	4
13	GCB extract	1	400	cellulose, rice bran, silica	2
14	GCB extract	2	600	cellulose	2
15	GCB extract	2	1050	gelatin, maltodextrin, magnesium stearate, silica, sodium copper chlorophyllin, titanium dioxide	2
16	GCB extract	1	400	gelatin, rice hull extract	6
17	pure GCB extract	1	800	cellulose	2
18	GCB extract	1[a]	400	black pepper extract, gelatin, glycerin, water, coconut oil, beeswax, red cabbage, turmeric	3[a]
19	GCB extract	1	800	cellulose	2
20	GCB extract	2	800	cellulose	2
21	pure GCB extract	1	800	cellulose	2
22	GCB extract	1	400	gelatin, cellulose, silica, magnesium stearate	1
23	GCB extract with raspberry ketone	1	800	raspberry ketone, African mango extract (*Irvingia gabonesis*), acai fruit, resveratrol, apple cider vinegar (powder), kelp, grapefruit (powder), gelatin, calcium carbonate, magnesium stearate	2
24	GCB extract	1	400	rice powder, magnesium stearate, gelatin	1
25	GCB extract	2	800	water, hypromellose	2
26	GCB extract	1	800	raspberry ketone, African mango extract (*Irvingia gabonesis*), acai fruit, resveratrol, apple cider vinegar (powder), kelp, grapefruit (powder), gelatin, calcium carbonate, magnesium stearate	na[b]
27	pure GCB extract	1	400	water, hypromellose	2
28	GCB extract	1[c]	700	green tea leaf extract, *Panax ginseng* root, calcium, chromium dinicotinate glycinate	1[c]
29	pure GCB extract	1	800	cellulose	2
30	GCB extract	1	500	cellulose, magnesium stearate, silica	3
31	pure GCB extract	1	800	cellulose	2
32	GCB extract	2	800	gelatin, rice powder	2
33	pure GCB extract	2	800	gelatin, rice powder	2
34	GCB extract	2	800	cellulose	2
35	GCB extract	2	250	thiamin, L-carnitine, riboflavin, nicotinic acid, gelatin, cellulose, silica, magnesium stearate	4
36	GCB extract	2	800	gelatin, cellulose	4
37	GCB extract	1	200	rice flour, cellulose	2
38	GCB extract	1	400	cellulose	2
39	GCB extract with yerba mate	3	400	yerba mate leaf extract (*Ilex paraguariensis*), cellulose, gelatin, silica, magnesium stearate	3
40	GCB extract	2	800	gelatin, cellulose	4
41	GCB extract	2	800	cellulose	6
42	GCB extract	1	400	rice powder, magnesium stearate, gelatin	1
43	GCB extract	1	200	gelatin, cellulose, rice flour, magnesium stearate, titanium dioxide	2
44	GCB extract	1	433	cellulose	3
45	GCB extract	1[d]	800	none	3[d]
46	GCB extract	1	800	cellulose	3
47	GCB extract	1	800	cellulose	3
48	GCB extract	1	200	rice flour, cellulose, magnesium stearate	2
49	GCB extract	2	800	cellulose	2
50	GCB extract	2	800	cellulose, magnesium stearate	4

[a]Softgel. [b]na, information not provided on label. [c]Pack. [d]Scoop (800 mg).

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Table 2. Parameter Settings of the UHPLC-MS/MS Method

			transition 1 (quantitative)					transition 2 (confirmatory)					
analyte	RT (min)	ESI mode	m/z Q1	m/z Q3	DP (V)	CE (V)	CXP (V)	m/z Q1	m/z Q3	DP (V)	CE (V)	CXP (V)	transition 1-to-transition 2 intensity ratio
aflatoxin B_1	3.4	+	312.9	285.0	101	33	8	312.9	241.1	101	53	8	1.23
aflatoxin B_2	3.2	+	314.9	286.8	106	37	10	314.9	259.0	106	41	10	1.04
aflatoxin G_1	3.0	+	328.9	242.9	76	37	8	328.9	200.1	76	55	6	1.49
aflatoxin G_2	2.8	+	330.9	313.0	81	35	10	330.9	189.0	81	57	8	2.70
beauvericin	8.1	+	801.2	784.2	11	25	18	801.2	243.9	11	41	8	1.29
citrinin	3.6	+	250.9	233.0	61	23	8	250.9	204.9	61	37	8	7.50
cyclopiazonic acid	6.2	+	336.7	196.1	81	29	8	336.7	182.0	81	25	8	1.23
diacetoxyscirpenol	3.6	+	384.0	307.3	57	15	15	384.0	105.1	57	60	15	2.55
enniatin A	8.6	+	699.2	682.2	86	27	22	699.2	210.0	86	39	8	1.20
enniatin A_1	8.4	+	685.2	668.3	81	25	22	685.2	210.0	81	39	14	2.75
enniatin B	8.0	+	657.1	640.1	101	27	22	657.1	196.1	101	39	14	1.45
enniatin B_1	8.2	+	671.3	654.2	76	27	20	671.3	196.1	76	39	8	2.50
fumonisin B_1	4.5	+	722.4	704.3	90	41	16	722.4	334.1	90	53	12	1.11
fumonisin B_2	5.5	+	706.1	336.1	121	49	12	706.1	318.2	121	51	12	1.50
fumonisin B_3	5.0	+	706.1	336.1	121	49	12	706.1	318.2	121	51	12	2.00
gliotoxin	3.5	+	326.7	262.9	56	15	10	326.7	245.0	56	25	10	1.55
HT-2 toxin	5.1	+	442.0	425.1	36	17	14	442.0	117.0	36	25	10	1.83
mycophenolic acid	4.7	+	320.9	207.0	71	29	6	320.9	303.0	71	13	10	1.03
neosolaniol	2.3	+	399.9	305.0	16	17	10	399.9	184.9	16	25	6	1.42
ochratoxin A	5.5	+	403.9	238.9	71	33	8	403.9	357.9	71	21	12	1.67
ochratoxin B	4.5	+	369.9	204.9	61	27	6	369.9	186.9	61	47	14	2.13
penicillic acid	2.3	+	170.9	125.0	51	17	10	170.9	153.0	51	11	8	1.25
penitrem A	7.1	+	634.01	558.0	51	27	16	634.01	616.2	51	47	14	4.70
roquefortin C	4.0	+	390.0	193.0	6	37	6	390.0	322.0	6	29	10	1.36
sterigmatocystin	5.7	+	325.1	281.1	85	48	15	325.1	310.1	85	33	17	1.42
T-2 toxin	4.9	+	484.0	305.0	26	19	10	484.0	215.0	26	23	6	1.38
tentoxin	4.5	+	415.0	312.1	96	29	10	415.0	256.0	96	41	8	1.70
3-acetyl-deoxynivalenol	3.7	−	397.1	306.9	−40	−19	−8	397.1	336.9	−40	−10	−18	2.00
alternariol	4.1	−	256.9	213.0	−120	−32	−11	256.9	214.9	−120	−35	−12	1.77
alternariol-methyl ether	4.6	−	271.0	255.9	−115	−30	−13	271.0	228.0	−115	−40	−15	3.33
deoxynivalenol	2.2	−	355.1	295.1	−42	−14	−7	355.1	265.1	−42	−18	−15	1.17
deoxynivalenol-3-glucoside	2.1	−	517.1	456.9	−55	−20	−22	517.1	426.9	−55	−25	−20	1.17
nivalenol	1.8	−	371.1	311.1	−45	−15	−8	371.1	281.0	−45	−18	−16	1.08
zearalenone	4.2	−	317.1	131.1	−71	−40	−10	317.1	175.0	−71	−30	−10	1.20

The chromatographic separation was carried out using a 100 mm × 2.1 mm i.d., 1.7 μm particle size, Acquity UPLC HSS T3 reversed phase analytical column (Waters, Milford, MA, USA) maintained at 40 °C. The injection volume was 3 μL. The autosampler temperature was maintained at 10 °C. Different aqueous mobile phases (A) were employed depending on the polarity setting of the ESI source. For analyses conducted in positive ion mode, (A) was composed of aqueous 5 mM ammonium formate with 0.2% formic acid; for those conducted in negative ion mode, (A) was composed of 5 mM ammonium acetate. The mobile phase (B) was methanol. A gradient program was used for separation of the analytes: 5% mobile phase (B) to 50% mobile phase (B) from 0 to 1 min, 50% mobile phase (B) to 100% mobile phase (B) from 1 to 8 min, held at 100% mobile phase (B) from 8 to 10 min, followed by column re-equilibration with 5% (B) from 10 to 13 min. The flow rate was set at 0.4 mL/min.

The 4500 QTRAP was operated in scheduled multiple reaction monitoring (MRM) mode using either positive or negative ESI. Two separate analyses were performed at each polarity. ESI settings were as follows: (i) positive mode, needle voltage, +4500 V; curtain gas, 35 psi; nebulizer gas (gas 1) and turbo gas (gas 2), 55 psi; turbo gas temperature, 500 °C; (ii) negative mode, needle voltage, −4500 V; curtain gas, 35 psi; nebulizer gas (gas 1) and turbo gas (gas 2), 55 psi; turbo gas temperature, 450 °C. The entrance potential (EP) was ±10 V. Optimal settings for declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP), were obtained for individual analytes during tuning. For identification, the intensity ratios of the two MRM transitions in samples were compared with those observed in the records of solvent standards. The maximum permitted tolerance for this ratio was ±20%.[27] The MRM transitions, intensity ratios, and compound-dependent settings used are summarized in Table 2.

The Q Exactive detector was operated in full MS data-dependent MS/MS mode with the following positive ESI settings: spray voltage, +3500 V; sheath gas, 35 arbitrary units; aux gas, 10 arbitrary units; capillary temperature, 350 °C; heater temperature, 250 °C. Full mass spectra were acquired at mass resolving power of 70000 full width at half-maximum (fwhm) in the range m/z 50−1100 without use of any lock masses. Data-dependent acquisition of tandem mass spectra was triggered automatically using an inclusion list that comprised information on m/z values and retention times (RT) of ochratoxin A ($[M + H]^+$ ion, m/z 404.0896, RT 5.7 min), ochratoxin B ($[M + H]^+$ ion, m/z 370.1285, RT 4.6 min), fumonisin B_1 ($[M + H]^+$ ion, m/z 722.3958, RT 4.7 min), and mycophenolic acid ($[M + NH_4]^+$ ion, m/z 338.1599, RT 4.9 min). Fragmentation mass spectra were recorded at a mass resolving power of 35000 fwhm with the use of a

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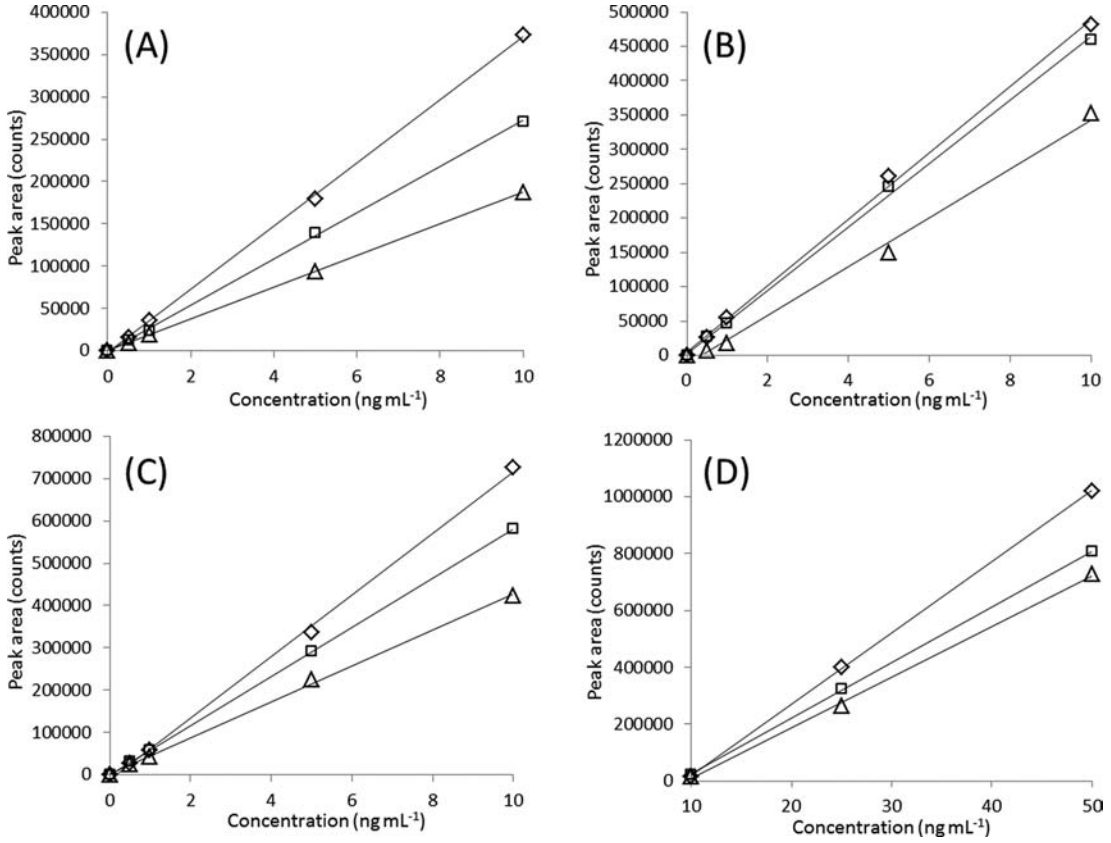

Figure 2. Matrix-matched calibration curves of ochratoxin A (A), ochratoxin B (B), mycophenolic acid (C), and fumonisin B_1 (D), prepared by spiking blank test solutions [product 6 (◇), product 20 (□), product 26 (△)].

normalized collision energy (NCE) of 20% and a quadrupole isolation window of 4 Da.

Evaluation of the UHPLC-MS/MS Method. The performance characteristics of the method were estimated by spiking products in which mycotoxins were not detectable. The recoveries were assessed at two concentrations of 20 and 100 μg/kg in six replicates using products 20 and 26. In the case of aflatoxins and ochratoxins, spikes at a concentration of 5 μg/kg were also analyzed. Additionally, quality control spikes at 20 μg/kg were prepared for each of the test materials. The spiked matrix was thoroughly mixed and incubated for 2 h under ambient conditions prior to extraction.

The matrix effects in QuEChERS extracts of dietary supplements were evaluated based on slopes of matrix-matched calibration curves. For this purpose, three extracts of blank test materials (products 6, 20, and 26) previously screened for target analytes were spiked with mycotoxins in the concentration range 0.1−50 ng/mL (corresponding to 1−500 μg/kg in matrix). Limits of detection (LODs) and limits of quantitation (LOQs) were estimated as the lowest matrix-matched calibration standards providing signal-to-noise ratios greater than 10 and 3, respectively, at both quantitative and qualitative transitions and matching the intensity ratio observed for the particular compound in the standard solution.[28] The method of standard additions was applied to evaluation of recoveries and quantitation of analytes in positive products. Volumes of standards containing 1, 5, and 10 ng of analytes were transferred to individual autosampler vials and evaporated to dryness. The residues of standard solutions were dissolved in 1 mL of products extract obtained by the above procedure. All four test solutions were analyzed by UHPLC-MS/MS. The peak areas of analytes (*y*-axis) were plotted versus the added concentrations (*x*-axis) and regression equations were obtained. The concentrations in the test solutions were back-calculated to be equal to the intercept of the regression line with the *x*-axis.

RESULTS AND DISCUSSION

Evaluation and Performance Characteristics of the UHPLC-MS/MS Method. Matrix-induced suppression or enhancement of analyte signals represents a major drawback that complicates quantitation in LC-MS-based analysis of complex biological materials.[29] In the initial phase of the evaluation of the method, the influence of the matrix on the signals of target mycotoxins was investigated in extracts of three dietary supplements differing in content of GCB extract and other ingredients. Without exception, signal suppression was observed for all analytes in all of the examined matrices. The peak areas of mycotoxins in QuEChERS extracts (spiked at a concentration of 10 ng/mL) were 40−89% lower than those measured in neat acetonitrile. More importantly, among tested matrices, the extent of matrix effects differed significantly for most of the analytes. Matrix-matched calibration curves for selected mycotoxins are provided in Figure 2. For example, in the case of ochratoxin A, based on three different matrix-matched calibration curves prepared from products 6, 20, and 26, the concentrations calculated for a peak with an area of 50000 counts were 14.0, 18.6, and 26.7 μg/kg, respectively. It was clear that a single matrix-matched calibration could not be used for accurate quantitation of target mycotoxins in all of the tested products.

Strategies based either on use of isotope-labeled internal standard or use of the method of standard additions are available to overcome the problems with quantitation caused by matrix effects.[29,30] Because the former approach could not be used due to lack of availability of isotope-labeled analogues for all analytes, the method of standard additions was employed.

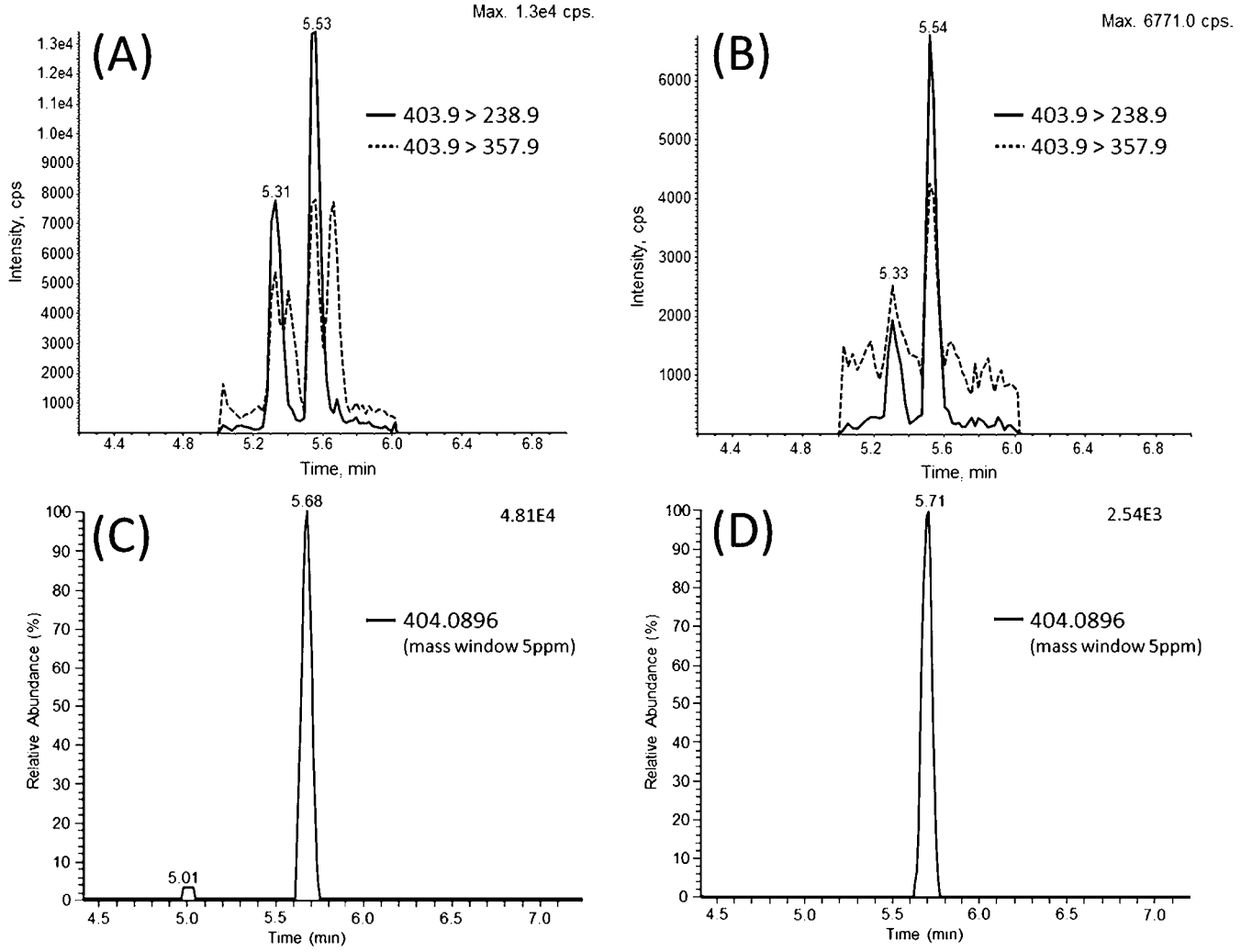

Figure 3. Confirmation of ochratoxin A in products 18 (22.9 μg/kg) and 23 (2.7 μg/kg) by UHPLC-HRMS: (A) UHPLC-MS/MS chromatogram of product 18; (B) UHPLC-MS/MS chromatogram of product 23; (C) UHPLC-HRMS chromatogram of product 18; (D) UHPLC-HRMS chromatogram of product 23.

Standards were added at three concentrations. Regardless of the analyte and sample, linear regression coefficients (R^2) higher than 0.98 were calculated for respective standard addition calibration curves.

Considering the lack of suitable reference materials, the accuracy of the method was characterized based on recovery analyses of spiked blank matrices comprising two products of differing GCB content. For most of the analytes, recoveries were in the range 75−109% and 78−110% for spiking concentrations of 20 and 100 μg/kg, respectively. The exception was deoxynivalenol-3-glucoside and fumonisins which showed recoveries of approximately 50%. Deoxynivalenol-3-glucoside (Figure 1, **7**), a metabolite of deoxynivalenol, represents a highly polar compound with high affinity for the aqueous phase. It cannot be completely transferred into the acetonitrile layer in the QuEChERS salt-induced separation of phases.[31] To improve poor recoveries of acidic fumonisins (Figure 1, **8**−**10**), the pH of the extraction solvent was decreased in order to prevent formation of anions. Additionally, water was added to the final acetonitrile extract in order to reduce possible binding of analytes to the glass surface of the autosampler vial.[25,32] Similar average recovery values were obtained in both tested matrices. The recoveries of mycotoxins calculated for quality control spikes (20 μg/kg) prepared for each of the test samples were consistent with those values.

The precision of the method was expressed as relative standard deviation calculated from results of six parallel sample analyses and was below 12% for all analytes. LODs and LOQs of mycotoxins ranged from 1.0 to 50.0 μg/kg and from 2.5 to 100.0 μg/kg, respectively.

Confirmation of Positive Results by HR-MS(/MS). Monitoring of two MRM transitions typically provides sufficient information to confirm target analyte identities within an LC-MS/MS analysis. However, when analyzing crude extracts of complex samples, the presence of matrix interferences on either one or both MRM transitions can result in false positive/negative results. To allow unequivocal confirmation of the results obtained by the UHPLC-MS/MS technique, samples testing positive were subjected to an additional analysis using the Q Exactive quadrupole-orbitrap mass spectrometer.

The criteria for identity confirmation were at least three scans at the retention time (RT) of the particular analyte in the extracted ion chromatogram using a 5 ppm mass window.[33] The presence of all target mycotoxins was successfully confirmed on the basis of accurate mass measurements and

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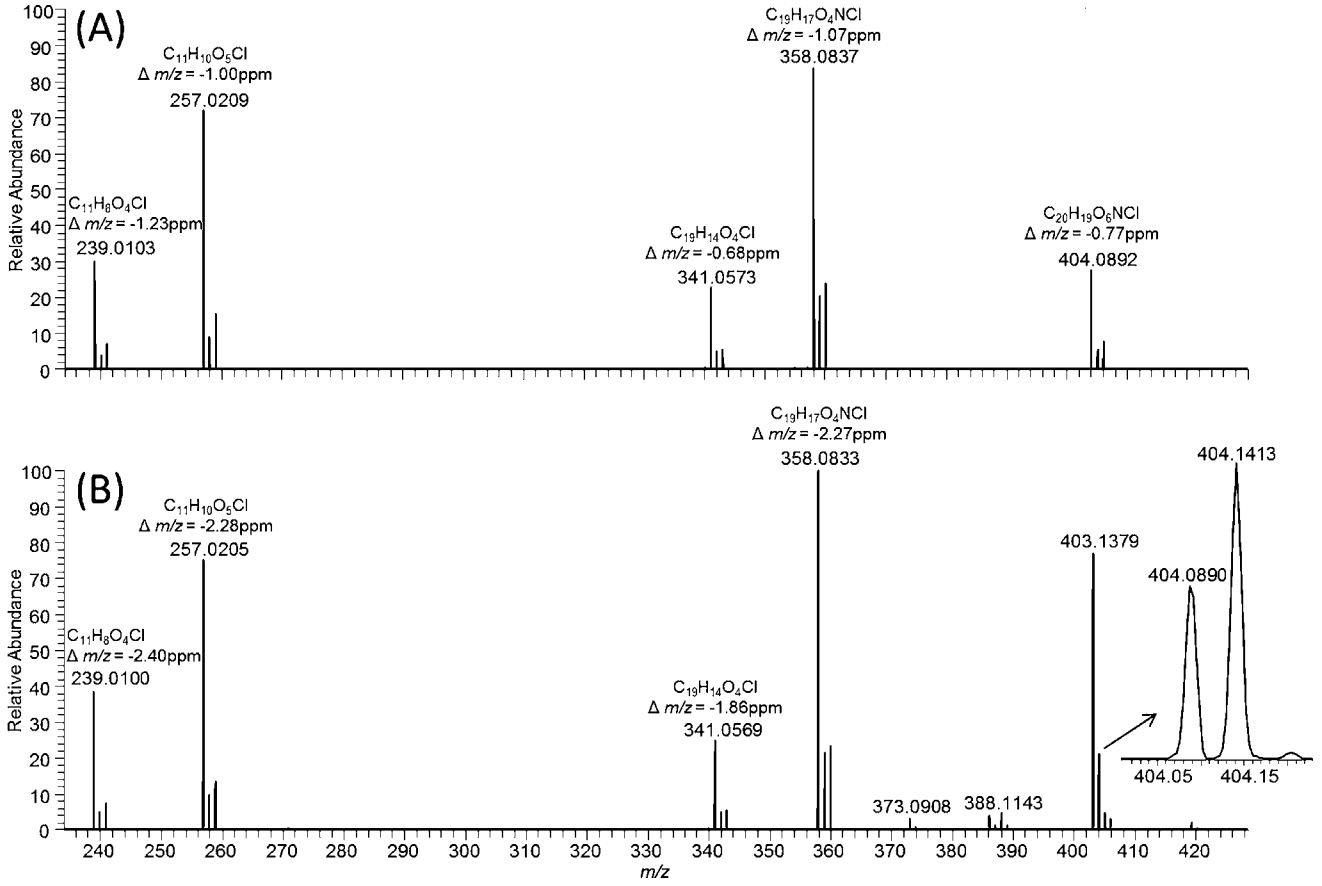

Figure 4. Data-dependent HR-MS/MS spectra of ochratoxin A: (A) record obtained by analysis of solvent standard (10 ng/mL); (B) record obtained by analysis of extract of product 18 (22.9 μg/kg).

comparison of RT with standards in all positive products. Without exception, mass errors below 4 ppm were obtained. Figure 3 documents the confirmation and substantial improvement of selectivity for ochratoxin A in products 18 and 23 as facilitated by HRMS.

In addition to full MS data acquisition, an MS/MS experiment was initiated during the analysis if a precursor ion was detected at a specified RT window with sufficient intensity. The availability of this additional confirmation was largely dependent on the concentration of the particular analyte in the sample extract. At very low concentrations of the targeted mycotoxin, the MS/MS experiment was either not triggered or the fragmentation spectra did not match the record obtained for the solvent standard. Therefore, the confirmation of ochratoxin A, ochratoxin B, fumonisin B_1, and mycophenolic acid based on HR-MS/MS spectra was possible only in products 16, 2, 3, and 5, respectively. In this case, the criterion was agreement with record of standard in m/z values of at least two fragment ions within 5 ppm. Figure 4B shows HR-MS/MS spectra obtained for ochratoxin A in product 18 (22.9 μg/kg). The fragmentation pattern acquired from the test solution matched both the spectra of the standard solution (Figure 4A) and was also in agreement with information provided in the literature.[34] The high mass resolving power of 35000 fwhm used in the MS/MS mode enabled resolution between parent ion of ochratoxin A (observed at m/z 404.0890) and isotope signal of a coeluting interference.

Occurrence of Mycotoxins in Commercial Products. The UHPLC-MS/MS method was used for screening of mycotoxins in fifty dietary supplements containing GCB extracts. The concentrations of mycotoxins in positive products were subsequently determined by use of the method of standard additions as described above. Among 34 targeted compounds, four mycotoxins, namely ochratoxin A, ochratoxin B, fumonisin B_1 and mycophenolic acid (Figure 1, **11**) were found in examined products in concentration ranges of <1.0 (LOD)−136.9 μg/kg, < 1.0 (LOD)−20.2 μg/kg, < 50.0 (LOD)−415.0 μg/kg, and <5.0 (LOD)−395.0 μg/kg, respectively. Ochratoxin A was present in 18 of 50 products (incidence 36%). Consistent with the information available in the literature,[18] ochratoxin B, a nonchlorinated structural analogue of ochratoxin A, co-occurred exclusively with this toxin and was detected in 16 of 18 positive products. The incidence of fumonisin B_1 (10%) was significantly lower compared to that of both ochratoxins A and B. Additionally, the concentrations of fumonisin B_1 in all tested materials were well below the EU maximum level. Although no limit has been set for GCB in the EU, the sum of fumonisins B_1 and B_2 is regulated in cereals at concentrations ranging from 800 to 4000 μg/kg, depending on the commodity type.[35] Mycophenolic acid was detected in 16% of tested samples. This mycotoxin, produced by several *Penicillium* species, represents a potent immunosuppressive compound in animals and humans.[36] The occurrence of mycophenolic acid has been reported previously only in feed (grain, silage, and grass) and foodstuffs such as bread, milk, cheese, and fruit.[37] Thus, to the best of our knowledge, this is the first report on its occurrence in GCB.

It is noteworthy that the two most contaminated supplement products (25 and 27) contained all detected mycotoxins at very similar concentrations (ochratoxin A, 136.9 and 128.0 μg/kg; ochratoxin B, 20.2 and 17.0 μg/kg; fumonisin B_1, 387.0 and 415.0 μg/kg; mycophenolic acid 227.3 and 217.0 μg/kg). Additionally, taking into account the almost identical average mass of the capsule contents (0.40 and 0.42 g), the two products were also matched in terms of content of GCB extract and other ingredients. This indicates that although offered by different vendors, both dietary supplements may have been produced from similar contaminated raw material. The occurrence of mycotoxins in the dietary supplements containing GCB extracts is summarized in Table 3.

Table 3. Occurrence of Ochratoxin A, Ochratoxin B, Fumonisin B_1, and Mycophenolic Acid in Dietary Supplements Containing GCB

product	ochratoxin A (μg/kg)	ochratoxin B (μg/kg)	fumonisin B_1 (μg/kg)	mycophenolic acid (μg/kg)
3	6.2	6.0	110.0	nd[a]
6	23.0	3.5	nd	nd
8	11.5	4.4	nd	nd
9	17.3	5.2	nd	nd
10	13.8	4.2	nd	nd
11	12.4	3.7	nd	43.1
14	16.3	4.0	nd	76.6
16	9.6	nd	322.0	nd
18	22.9	3.6	nd	nd
22	16.9	7.5	241.0	395.0
23	2.7	nd	nd	nd
25	136.9	20.2	387.0	227.3
27	128.0	17.0	415.0	217.0
31	62.1	6.1	nd	82.9
36	47.7	6.3	nd	nd
39	26.2	5.1	nd	nd
40	54.1	4.2	nd	51.6
44	61.5	5.9	nd	66.6
incidence (% of all tested samples)	36	32	10	16

[a]nd, not detected.

The intake of ochratoxin A and fumonisin B_1 from dietary supplements testing positive for these mycotoxins was calculated in the final phase of the study. The tolerable weekly intake (TWI) of 120 ng/kg body weight per week and tolerable daily intake (TDI) of 2000 ng/kg body weight per day were estimated by the EU Scientific Committee on Food (SCF) and the European Food Safety Authority (EFSA) for ochratoxin A and fumonisin B_1, respectively. These values were used since comparable US values are not available. Considering the daily dosage recommended by the supplier and the average masses of respective dosage forms, the intake of ochratoxin A from examined samples ranged from 0.1 up to 8.0% of TWI (calculated for an adult of 80 kg). In the case of fumonisin B_1, the intake percentage was in the range 0.0−0.7% of TDI.

A UHPLC-MS/MS method for simultaneous determination of 34 mycotoxins in GCB extract dietary supplement products was developed and evaluated. The method employed a rapid and simple sample procedure and used the method of standard additions to overcome problems with quantitation caused by matrix effects in tested products. Quantitative testing of fifty dietary supplements revealed the presence of ochratoxin A, ochratoxin B, fumonisin B_1 and mycophenolic acid with incidence rates ranging from 10 to 36%. The results of the survey show the necessity for monitoring the quality of GCB extracts with respect to these natural contaminants. Additionally, the usefulness of UHPLC-HR-MS operated in full MS data-dependent MS/MS mode for confirmation of mycotoxins in complex crude extracts was demonstrated.

■ ASSOCIATED CONTENT

Supporting Information

Table S-1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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